Canine leptospirosis in Switzerland—A prospective cross-sectional study examining seroprevalence, risk factors and urinary shedding of pathogenic leptospires

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Canine leptospirosis in Switzerland – A prospective cross-sectional study examining seroprevalence, risk factors and urinary shedding of pathogenic leptospires

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Running title: Seroprevalence and urinary shedding of *Leptospira* in dogs

Abstract

Leptospirosis is an important worldwide zoonosis. While human leptospirosis remains rare in Switzerland, the incidence of canine leptospirosis is unusually high compared to other European countries. The aims of this cross-sectional study were to determine the

exposure of asymtomatic dogs to pathogenic *Leptospira* in Switzerland, to characterise risk factors associated with seropositivity and to determine the prevalence of urinary shedding. Sampling was stratified to cover the whole of Switzerland. Sera were tested by microscopic agglutination test for antibodies against a panel of 12 serovars. Urine was tested for pathogenic *Leptospira* using a *LipL32* real-time PCR. Of 377 sera, 55.7% (95%CI 51.2-60.7) showed a reciprocal MAT titre of ≥1:40 and 24.9% (95%CI 20.7-29.4) of ≥1:100 to at least one serovar. Seropositivity (MAT ≥1:100) was most common to serovars Australis (14.9%; 95% CI 11.4-18.6) and Bratislava (8.8%; 95%CI 6.1-11.7), followed by Copenhageni (6.1%; 95%CI 3.7-8.5), Canicola (5%; 95%CI 2.9-7.4), Grippotyphosa (4.5%; 95%CI 2.7-6.9), Pomona (4%; 95%CI 2.1-6.1), Autumnalis (2.7%; 95%CI 1.3-4.2) and Icterohaemorrhagiae (1.6%; 95%CI 0.5-2.9). In unvaccinated dogs (n=84) the prevalence of a MAT titre ≥100 was 17.9% (95%CI 10.7-26.2), with a similar distribution of reactive serovars. Variables associated with seropositivity (≥1:40) to any serovar included age (OR 1.29/year; 95%CI: 1.1-1.5) and bioregion with higher risks in the regions Northern Alps (OR 14.5; 95%CI 2.2-292.7), Central Plateau (OR 12.3; 95%CI 2.0-244.1) and Jura (OR 11.2; 95%CI 1.7-226.7) compared to Southern Central Alps. Dogs living with horses were significantly more likely to have antibodies to serovar Bratislava (OR 4.68;95%CI 1.2-17.2). Hunting was a significant risk factor for seropositivtiy to serovar Grippotyphosa (OR 8.03; 95%CI 1.6-30.8). Urine qPCR positivity was uncommon (1/408 dogs; 0.2%; 95% CI0-0.7). These results demonstrate that dogs in Switzerland are commonly exposed to pathogenic *Leptospira;* however, the risk of dogs contributing to the spread of *Leptospira* in the environment appears low.

Keywords: *Leptospira*; ; ; ; ; , Microscopic agglutination test, real time LipL32 PCR, urinary shedding, risk factors, dog

1. Introduction

Leptospirosis is a zoonotic disease with worldwide distribution affecting most mammalian species (Bharti et al., 2003). It is caused by infection with pathogenic *Leptospira* spp. The organism maintains itself in nature by colonising the renal tubules of reservoir or maintenance hosts from which they are shed into the environment via urine. Maintenance hosts are often rodents, but in principle any host susceptible to infection can become a chronic carrier of *Leptospira*. Due to co-evolutionary adaptation, many serovars have a preference for specific maintenance hosts (e.g. rats often carry serovar Icterohaemorrhagiae) and cause very little clinical signs of infection in these hosts. In contrast, if a host gets infected with a non-adapted serovar (incidental host infection), acute and potentially life threatening disease can develop. Clinical manifestations in incidental hosts include fever, renal and hepatic injury, pulmonary haemorrhage and reproductive failure (Adler, 2015). In general, infected maintenance hosts show leptospiruria of higher intensity and longer duration compared to incidental hosts (Chernukcha et al., 1974; Rojas et al., 2010).

Human leptospirosis is a major public health issue in many countries, including Latin America and South-East Asia, with large outbreaks linked to natural disaster and flooding (Abela-Ridder et al., 2010). In developed countries of temperate zones leptospirosis is considered a rare disease, with an increased occupational risk for professions such as farmers, slaughterhouse workers, sewage workers and veterinarians and for individuals performing outdoor activities such as fishing and water sports (Picardeau, 2013).

In dogs, risk factors for acute clinical infection and exposure in asymptomatic dogs, as well as associations of seropositivity with environmental or climatic factors have been reported from a number of different areas of the world including Europe, North and South America; Australia and New Zealand with sometimes contradicting results. A

recent meta-analysis identified the following major risk factors for leptospirosis: male sex, mixed-breed, young dogs (<1 year), working dogs, flooding occurrence in the habitat of the dog and urban environment (Azocar-Aedo and Monti, 2016). Other possible risk factors are swimming, drinking from outdoor water and exposure to wild animals (Ghneim et *al.*, 2007).

The epidemiology of leptospirosis in Switzerland is incompletely understood. While 10-20% of mice, moles and shrews captured in the city of Zürich showed PCR- based evidence of renal colonisation with *Leptospira* spp, more specific information on serovars present in the environment and their relevant maintenance hosts is currently not available. While the prevalence and incidence of leptospirosis in humans is unknown due to low awareness of the disease and lack of an official notification system, a cluster of acute leptospirosis in young, previously healthy persons was recently identified in Switzerland and traced back to a surfing spot on the river Reuss in canton Aargau (Schreiber et al., 2015).

A dramatic increase in the incidence of canine leptospirosis has been documented in Switzerland between 2003 and 2012 with a peak incidence of 28/100.000 dogs in canton Aargau in 2012 (Major *et al.,* 2014). In that study over 75% of dogs, presented to a veterinary university hospital developed leptospiral pulmonary haemorrhage syndrome, a severe and often lethal form of leptospirosis, which has previously been considered rare. About 70% of dogs in this cohort showed serologic evidence of infection with serovars Bratislava and Australis, both belonging to serogroup Australis (Major et al., 2014). The natural reservoir for members of this serogroup in Switzerland has not yet been identified. While horses are considered an important maintenance host for serovar Bratislava (Arent et al., 2015), it has been suspected that this serovar

can be equally carried by asymptomatic dogs thus potentially acting as a source of infection for other domestic and wild animals and for humans (Ellis, 2010).

Urinary shedding of pathogenic *Leptospira* has been reported in 1.5-8% of dogs not suspected to have leptospirosis (Harkin et al., 2003; Rojas et al., 2010; Llewellyn et al., 2016). Dog ownership has been identified as a risk factor for human leptospirosis in Germany (Jansen et al., 2005), Barbados (Douglin et al., 1997) and Nicaragua (Trevejo et al., 1998) suggesting transmission of *Leptospira* spp from dogs to humans.

We hypothesised that there is a high level of exposure to pathogenic *Leptospira* in dogs in Switzerland. Furthermore we hypothesised that urinary shedding of leptospires is common in dogs without clinical signs of leptospirosis and that dogs may therefore contribute to environmental contamination.

The aims of this present study were: 1) to estimate the level of exposure to pathogenic *Leptospira* sp. by determining the prevalence of anti-leptospiral serum antibodies, 2) to determine the prevalence of urinary shedding of pathogenic leptospires and 3) to determine risk factors for exposure to pathogenic leptospires including age, sex, breed, geographic area, vaccination status and lifestyle in dogs not suspected to have leptospirosis in Switzerland.

2. Material and Methods

2.1. Ethical approval

Ethical approval was granted by the Federal Food Safety and Veterinary Office for the study protocol (Project BE9/15). Written owner consent was obtained before enrolment of dogs in the study.

2.2. Study design, recruitment, inclusion and exclusion criteria

The study was designed as a cross-sectional prevalence study representing the whole of Switzerland. Sampling was stratified based on the number of dogs per canton according to the Swiss national registry for dogs (ANIS database, Animal Identity Service AG, Bern, Switzerland). The sample size was calculated to estimate the true prevalence and a significant difference between two proportions assuming a prevalence of 10%, test sensitivity and specificity of 95%, a power of 85% and confidence levels of 0.05. The estimated prevalence was based on the results of previous studies (Arent et al., 2013; Schuller et al., 2015); (Davis et al., 2008); (Llewellyn et al., 2016).

Dogs were recruited at the Vetsuisse Faculty Bern and Zürich, as well as via 30 private practices throughout Switzerland between April and December 2015. Private practices were recruited during in-house conferences at the Vetsuisse Faculty Bern and Zürich. In areas with low participation, additional practices were contacted via phone and invited to participate.

Dogs presented for routine check-ups, vaccination or problems unrelated to leptospirosis were eligible for inclusion. Dogs had preferably not been vaccinated against leptospirosis during the previous 16 weeks and were excluded from urine real time PCR (qPCR) examination if they had received antibiotics within the past 4 weeks. Repeated MAT testing was advised in cases with reciprocal titres of ≥1:40 in order to document titre dynamics.

2.3. Sampling and Sample Handling

2.3.1. Stability of leptospiral DNA in canine urine with and without stabilizer

In preparation of the study the stability of leptospiral DNA in canine urine during simulated shipping at ambient temperature with and without a stabiliser (AssayAssure®; Sierra Molecular Corporation; USA) was tested. Fresh voided urine

from dogs without evidence of urinary tract infections as shown by absence of clinical signs, normal dipstick results, inactive sediment and negative bacterial culture were collected via free catch. Samples were pooled, split into 4 ml aliquots and spiked with *Leptospira interrogans* serovar Autumnalis at $1x10^4$/ml, $1x10^3$ and $1x10^2$/ml. The strain *L. interrogans* serovar Autumnalis Akiyami was chosen due to its intermediate detection limit (5 Genome equivalents per reaction) determined in a previous study (Stettler, 2015). Stabiliser was added to the test group at a concentration of 1:11 according to the manufacturer's instructions. The spiked samples were incubated at room temperature for 4, 24 or 48 hours and then processed for DNA extraction and real time PCR as described below. All spiked samples incubated for 4 and 24 hours showed amplification of the target gene *lipl32* regardless of the addition of stabiliser and incubation time. However, after incubation of 48 hours no positive amplification could be yielded without the addition of stabiliser **(Suppl. Table 1).** Based on these positive results, the stabiliser was used for all urine samples in this study.

2.3.2. Urine samples

Urine samples were collected via free catch unless cystocentesis was indicated for medical reasons. Four ml of urine were immediately transferred into polypropylene tubes prefilled with 0.36 ml of stabiliser (ratio 1:11).

2.3.3. Blood samples

Blood was collected into tubes without anticoagulant. Blood was either sent to the laboratory uncentrifuged or left to clot for 30 min before centrifugation at 2,000 x g for 10 minutes and subsequent serum separation.

2.3.4. Shipping and pre-analytical sample handling

Urine, whole blood or serum samples were shipped at ambient temperature to the Department of Veterinary Bacteriology of the Vetsuisse Faculty Bern, which hosts the

Swiss National Reference Laboratory for animal leptospirosis and is accredited by the Swiss Accreditation Service (SAS) for both techniques, MAT and real-time PCR. Quality management is according to DIN EN ISO/IEC 17025 with regular participation in the proficiency testing schemes of the National Serology Reference Laboratory (Australia) and Vetqas AHPA (UK) for serology and real-time PCR.

DNA extraction was performed within 48 h of sampling. Serum and DNA extracts were either immediately analyzed or frozen at -20°C until further analysis.

2.4. Questionnaire

Owners were encouraged to fill in a questionnaire addressing the following points: age, sex, breed, neuter status, occurrence of a febrile illness in the dog or owner in the past year, treatments in the past 4 weeks, life style including residential environment (urban, countryside, farm, presence or absence of a garden), whether the dog is mainly kept indoor or outdoor, contact to other pets, activities including drinking from puddles, swimming in natural waters, hunting, walking in the woods, contact with wild animal species and knowledge of confirmed cases of leptospirosis in the vicinity **(Supp. Figure 1).** The date of last vaccination and type of vaccine were either checked directly on the vaccination booklet by the veterinarian, or owners were asked by phone to read aloud the vaccination record.

2.5. Microscopic agglutination testing (MAT)

Sera were examined for the presence of antibodies against pathogenic *Leptospira* by microscopic agglutination test (MAT) according to OIE standards (Office International des Epizooties OIE, 2008).

For MAT testing, live cultures of twelve reference strains of *Leptospira* belonging to 10 serogroups were used **(Table 1).** Sera were initially screened for agglutination of *Leptospira* at a dilution of 1:25. Reactive sera were titrated in a serial two-fold dilution

to determine the end-point titre defined as the highest serum dilution at which at least 50% agglutination occurs. All samples were tested by the same person to avoid inter-observer variability.

2.6. DNA extraction from urine and Real time PCR

Urine samples were centrifuged at 10 000 rpm at 4°C for 10 min. Subsequently, the supernatant was discarded and the pellet resuspended in 100 µL lysis buffer (containing 180 ml pyrogen-free water, 20 ml TrisHCl 1M pH 8.5, 100 µL Tween 20 and 48 mg Proteinase K (Sigma-Aldrich Co, St. Louis, Missouri, USA) and incubated for 60 min at 60°C, followed by 15 min at 97° on an Eppendorf thermomixer comfort (Vaudaux-Eppendorf AG, Basel, Switzerland). The lysates were stored at -20°C.

The real-time PCR targeted the gene encoding leptospiral major outer membrane protein LipL32 which is only present in pathogenic *Leptospira* spp. Primers and probe were according to (Villumsen et al., 2012) using the following primers: LipL32F (5'-AGA GGT CTT TAC AGA ATT TCT TTC ACT ACC T-3'), LipL32R (5'- TGG GAA AAG CAG ACC AAC AGA-3') and LipL32-P (5'-FAM-AAG TGA AAG GAT CTT TCG TTG C-MGB-3'). The reactions were carried out on a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with the cycling settings recommended by the manufacturer in a total reaction volume of 25 µL containing: 12.5 µL TaqMan® Universal PCR Master Mix, No AmpErase® UNG at a concentration of 100 µM, a 1 µM concentration of each forward and reverse primer, a 80 nM concentration of the probe, 0.5x TaqMan® Exogenous Internal Positive Control (IPC) mix, 0.5x IPC template (all reagents Applied Biosystems, Foster City, CA, USA), and 2.5 µL of template. DNA from *Leptospira* interrogans serovar Icterohaemorrhagiae strain RGA was included as a positive control and pyrogen free water as a negative control. Values above the threshold of 0.06 at cycle times <40 were considered positive.

2.7. Statistical analyses

A graphical overview of dogs included in the different steps of the analysis is shown in **Figure 1**. Descriptive statistics and the prevalence calculations were performed using IBM SPSS Statistic Version 23 for Windows. Univariate and multivariate analyses were performed using R 3.2.2 (2016, R Core Team, Vienna, Austria).

MAT positivity was defined as positive reaction to at least one serovar included in the 12 serovar panel at reciprocal titres of ≥1:40 (ALL40) or ≥1:100 (ALL100). Two, instead of one cut-off titres were chosen in the absence of a consensus to what represents an ideal cut-off titre to document exposure in a population of dogs not suspected to have leptospirosis.

The prevalence of positive urine qPCR and MAT seropositivity were calculated with a confidence interval of 95%. In order to examine the possible effect of vaccination on MAT seropositivity, seroprevalence was calculated for the overall population as well as for the group of unvaccinated dogs (n=84) and the group of dogs not vaccinated within the past 16 weeks (n=255).

Risk factor analysis was restricted to MAT seropositivity as outcome measure because only one dog tested positive on qPCR. Risk factor analysis was performed using the whole study population and without exclusion of vaccinal serogroups (Icterohaemorrhagiae, Canicola, Australis, Grippotyphosa). To test for the potential effect of vaccination on MAT seropositivity, the variable “days since vaccination” was introduced into the analysis. For unvaccinated dogs a value had to be generated in order to incorporate them into the model and 700 days was regarded a period after which antibodies were present due to exposure to leptospires and not due to vaccination. In order to identify risk factors, the association of independent variables derived from the questionnaire and the newly generated variable “days since

vaccination" with MAT seropositivity at reciprocal titres of ≥1:40 (ALL40) or ≥1:100 (ALL100) were assessed. In a second step risk factors for seropositivity (MAT ≥1:100) was assessed for the serovars Australis (AUS100), Bratislava (BRA100), Grippotyphosa (GRI100), Canicola (CAN100) and Copenhageni (COP100), which were the serovars that sera most commonly reacted with. Variables included in the risk factor analysis are shown in **Table 2.**

Univariate analysis was performed using $\chi 2$ or Fisher's exact test for categorical variables and univariate logistic regression for continuous variables. A value of $p \leq 0.20$ was considered as the critical level of significance for a variable to be entered in a full multivariable logistic regression model. Subsequently, for each outcome a manual likelihood-ratio-test backward selection of exposure variables was used to determine the best fitting model and to determine independent potential risk or confounding factors associated with *Leptospira* seropositivity. Risk factors were maintained in the model at a p-value ≤ 0.05.

The assumption of independence of the data was potentially violated, since some dogs were owned by the same owner and thus were clustered. To correct for dependency, we explored extending the model by generalised estimating equations (GEE) to control for clustering, by expanding the standard errors (SE) ('robust SE') and increasing the p-values. An independence and exchangeable pattern was used as correlation structure (Liang, 1986). However, the SE hardly changed and therefore the results of unadjusted multivariable logistic regression models were reported. The final models were evaluated on goodness of fit using the Hosmer-Lemeshow test. The models showed no evidence of lack of fit and there was no strong leverage. All observations were therefore kept in the model. Influential observations were detected using Pearson residuals, hat matrix (leverage) and delta-betas (Hosmer, 2004).

For analysis of geoclimatic factors on seroprevalence, the Swiss Territory was categorized into six bioregions according to a classification proposed by the Swiss Federal Office for the Environment (BAFU) (Gonseth, 2001). The six regions were Jura, Central Plateau, Northern Alps, Central Western Alps, Central Eastern Alps and Southern Alps; each bioregion presenting similar geological, geomorphological and climatic conditions **(Figure 2)**.

2.8. Mapping

Maps were created using QGIS 2.14 (QGIS Development Team, 2016. QGIS Geographic Information System. Open Source Geospatial Foundation Project)

3. Results

3.1. Study population

The sample size to estimate a true prevalence and to identify risk factors with sufficient statistical power was estimated to be 229 and 536 dogs, respectively.

The majority of dogs included in this study were privately owned pet dogs. Eleven dogs were owned by the military, but lived in individual homes with their handlers. 21 dogs lived in two different rehoming kennels.

Of the 377 dogs tested by serology, 21.1% lived in urban areas and 73.1% in rural environments including 16.9% farm dogs.

Dogs originated from 20 of the 26 Swiss cantons. The geographical distribution of the sampled dogs across Switzerland is shown in **Figure 3.**

The age of the dogs ranged from 4 months to 15 years (median 5 years IQR 2-9). Of the 423 dogs of known breed, 341 (80.6%) were purebred from 102 different breeds and 82 dogs (19.3%) were mixed-breeds. The most common breeds were Malinois (8.3%), Labrador Retriever (7.8%), French Bulldogs (4%), Golden Retrievers (3.5%)

and Jack Russel Terrier (3.3%). The overrepresentation of Malinois dogs was due to the inclusion of military dogs as well as dogs of this breed undergoing genetic screening. Gender was known in 97.8% of dogs and was equally distributed with 204 (45.3%) female dogs (57 entire/146 neutered) and 246 (54.7%) male dogs (139 entire/107 neutered).

Of the 377 dogs tested by MAT, 373 had a known vaccination history: 43 dogs (11.6%; 95%CI 40.6-50.7) were vaccinated using a bivalent vaccine including serovars Canicola, and Icterohaemorrhagiae, 246 (66.3%; 95%CI 61.2-70.9) with a quadrivalent vaccine including serovars Canicola, Grippotyphosa, Icterohaemorrhagiae and Pomona and 84 dogs (22.1%; 95%CI 18.1-26.4) were not vaccinated. The median period between vaccination and test was 252.5 days (IQR 161-342).

3.2. Urinary shedding

qPCR of urine was performed for 408 dogs without clinical suspicion of leptospirosis. Only one dog had a positive PCR result. This dog lived on a farm in Canton Vaud and reportedly had a habit of chasing rats. The dog showed reciprocal MAT titres of 1:40 to serovar Bratislava and 1:20 to serovars Australis, Canicola, Hardjo and Autumnalis. The dog had been vaccinated with a bivalent anti-leptospiral vaccine 93 days before sampling.

In 12 dogs qPCR was repeated 2-4 weeks after initial testing because of MAT titres ≥1:40 to one or several serovars. All the repeated urine samples tested negative.

3.3. Seroprevalence and Serovars

Due to the potential effect of vaccination on MAT seroreactivity is shown for the overall population as well as according to the vaccination status of the dogs in **Figure 4 A-D.** While reciprocal tires of ≥1:40 and ≥1:100 were chosen as cut offs for risk factor

analysis, the prevalence of MAT seroreactivity was calculated for titres between ≥1:20 and ≥1:3200 and are shown in **Tables 3-4 and Suppl. Tables 3-4.**

3.3.1. Overall population

Of 377 sera, 55.7% (95%CI 51-60.7) showed a reciprocal MAT titre of ≥1:40 and 24.9% (95%CI 20.7-39.4) of ≥1:100 to at least one serovar.

At ≥1:100, seropositivity was most common to serovars Australis (14.9%; 95%CI 11.4-18.6) and Bratislava (8.8%; 95%6.1-11.7), folowed by Copenhageni (6.1%; 95% 3.7-8.5), Canicola (5%; 95%2.9-7.4), Grippotyphosa (4.5%; 95% 2.7-6.9), Pomona (4%; 95%2.1-6.1), Autumnalis (2.7%; 95%1.3-4.2) and Icterohaemorrhagiae (1.6%; 95%0.5-2.9).

At ≥1:40 the reactivity pattern was slightly different with Australis (32.6; 95%CI 28.1-32.6, Canicola (24.9; 95%CI 20.7-29.4), and Copenhageni (18.3; 95%CI 14.3-22.5) being the most commonly reactive serovars, followed by Bratislava (14.3; 95%CI 10.6-18), Grippotyphosa (11.4; 95%CI 8.2-14.9), Icterohaeorrhagiae (9.5; 95%CI 6.9-12.7) and Pomona (9.8; 95%CI 6.9-13).

In order to better understand the differences in reactivity, co-reactiviy patterns were also analysed **(Table 5).** The number of dogs with MAT reactivity to more than one serogroup was higher at a cut-off titre of ≥1:40 than at a cut-off titre of ≥1:100. The most common serogroup co-reactivities or double exposures at a MAT cut-off of ≥1:100 were serogroup Australis and Icterohaemorragie (40%), Australis and Grippotyphosa (28.6%), Australis and Pomona (28.6%) and Canicola and Icterohaemorrhagie (25.7%).

3.3.2. Dogs not vaccinated in the past 16 weeks.

Of 339 sera, 52,8% (95%CI 47.5-58.1) were reactive at a MAT titre of ≥1:40 and 22.7% (95%CI 18-27.4) of ≥1:100 to at least one serovar. Seropositivtiy (MAT ≥1:100) was most

common to serovars Australis (13%; 95%CI 9.7-16.5), Bratislava (8.3%; 95%CI 5.6-11.5), Canicola (4.7%; 95%CI 2.7-7.1), Copenhageni (4.7%; 95%CI 2.7-7.1), Grippotyphosa (1.5%; 95%CI 2.4-6.8), Pomona (3.5%; 95%CI 1.8-5.6), Autumnalis (2.9%; 95%CI1.5-5) and Icterohaemorrhagiae (1.5%; 95%CI 0.3-2.9).

3.3.3. Unvaccinated dogs.

In unvaccinated dogs (n=84) the overall seroprevalence was 44% (95%CI 33.3-53.6) at a reciprocal MAT titre of ≥1:40 and 17.9% (95%CI 10.7-26.2) at a reciprocal MAT titre of ≥1:100. The distribution of serovars was largely similar to the distribution in vaccinated dogs with Australis (9.5%; 95%CI 3.6-16.6), Bratislava (8.3%; 95%CI 2.4-14.3) being the most frequent serovars, followed by Copenhageni (3.6%; 95%CI 0-8.3), Grippotyphosa (3.6%; 95%CI 0-8.3), Canicola (3.6 95%CI 0-8.3), Pomona (2.4%; 95%CI 0-6) and Autumnalis (2.4%; 95%CI 0-6).

3.3.4. MAT titre dynamics

Despite the fact that in the study protocol retesting was advised in dogs with reciprocal MAT titres ≥1:40, only 11 dogs were retested after 2-3 weeks; one reason being that many dogs were sampled for this study immediately prior to vaccination and thus no meaningful information could be derived from repeat MAT testing due to the interference of vaccinal titres. One dog showed seroconversion (BRA: 1:400 to 1:1600) and one dog showed persistently high titres (BRA: 1:800 to 1:1600; AUT: 1:1600 to 1:1600), which can be consistent with recent infection. Four dogs showed decreasing titres and five dogs the same or a slightly higher titre as before.

3.4. Risk factor analysis and multivariable models

The prevalence of anti-leptospiral serum antibodies by sex, gender, neuter status, vaccination status, region and lifestyle is reported in **Suppl Table 2.**

3.4.1. Univariate analysis

The association of 17 variables for the outcomes MAT seropositivity at titres ≥40 or ≥100 to any serovar or ≥100 to individual serovars were assessed via univariate analysis. The 10 variables with significant (p≤ 0.2) associations with the different outcomes in the univariate analysis are shown in **Table 6**.

3.4.2. *Multivariable analysis*

The fitted models after backwards selection based on the likelihood ratio test are displayed in **Tables 7 and 8.**

Dogs living in the bioregions Jura, Central Plateau. Northern Alps and Central Eastern Alps had significantly higher odds of being seropositive for any serovar compared to the bioregion Southern Alps.

The odds of seropositivity to any serovar significantly increased with age at both cut off titres ≥1:40 and ≥1:100. The interaction between “Age” and “Days since vaccination” was significant for both cut off titres, meaning that the effect of age on seropositivity differed according to the amount of days between vaccination and test.

Being castrated significantly increased the odds of seropositivity against serovar Australis (MAT ≥1:100)**.** The odds of seropositivity with the serovar Bratislava (MAT≥ 1:100) significantly increased when the household owned a horse.

Hunting significantly increased the odds to be seropositive for serovar Grippotyphosa. For the serovars Canicola and Copenhageni no risk factors remained significant**.**

4. Discussion

4.1. Seroprevalence

The results of this stratified cross-sectional study demonstrate that exposure to pathogenic *Leptospira* is common in dogs not suspected to have leptospirosis in Switzerland. Of 377 dogs tested, 24.9% showed seroreactivity to one or several

serovars of a 12 serovar MAT panel at a cut-off titre of ≥100 and 55.7% of dogs showed reactivity at a titre of ≥40. The seroprevalence of anti-leptospiral antibodies in asymptomatic dogs reported in the literature ranges between 6% in dogs in Ireland using a MAT cut-off of ≥1:10 (Schuller et al., 2015), 11.4% in Greece (Burriel et al., 2003), 17% in dogs in southern Germany (Llewellyn et al., 2016), 17.1% in Washington State, 21.5% in Poland (Krawczyk, 2005) and 49% in kenneled dogs in Italy using all a cut off ≥1:100 (Scanziani et al., 2002). In comparison, the overall seroprevalence of 24.9% in Switzerland does not appear exceedingly high, especially in view of the very high incidence of acute disease in this region in the past years (Major et al., 2014). A possible explanation is that albeit dogs do not seem to get more commonly exposed to pathogenic *Leptospira*, serovars in circulation may be particularly virulent causing acute and severe disease in those dogs that do get infected. Another potential explanation could be that we are already seeing a change in epidemiology of canine leptospirosis in Switzerland due to the introduction of new quadrivalent vaccines, by the end of 2014. These vaccines contain serovars belonging to the serogroups Australis and Grippotyphosa in addition to serogroups Canicola and Icterohaemorrhagiae and thus potentially provide a broader spectrum of protection.

4.2. Reactive serogroups

Serovars Australis and Bratislava, both belonging to serogroup Australis, were associated with about half of the reactive MATs, regardless of the vaccination status of the dogs. These findings correlate with the fact that based on serological data, members of serogroup Australis are an important cause of acute clinical infection in dogs in Switzerland (Major et al., 2014). However, canine sera also reacted with a broad range of other serovars including Copenhageni, Canicola, Grippotyphosa, Pomona, Autumnalis and Icterohaemorrhagiae indicating that *Leptospira* belonging to these serovars are in circulation in the environment. Studies are underway to examine

the role of wild animals as reservoir hosts for specific serogroups in Switzerland. Serovar Bratislava has been isolated from hedgehogs in Ireland (Hathaway et al., 1983) and have been suggested as potential reservoir of serovars belonging to serogroup Australis in France (Ayral et al., 2016). Hedgehogs are frequent visitors in private gardens, and thus can come into close contact with residing dogs. The role of hedgehogs as reservoir host for serovars belonging to serogroup Australis in Switzerland should therefore be further investigated.

4.3. Risk factors

In order to assess areas of increased risk, the prevalence amongst 6 different biogeographic regions was compared. This bioregion classification is based on the flora and fauna found in different areas of Switzerland and reflects the significant climatic variations between lower Switzerland (Plateau), with low to moderate altitudes (230- 960 m NN), the Jura with medium to high altitudes (360-1300 m NN), the northern and southern slopes of the Alps and the high alpine areas with altitudes of up to 4600 m NN (Gonseth, 2001). This classification does not take into account the potentially significant regional variations in microclimate, as well as other factors of importance to the transmission of *Leptospira* such as the presence of stagnant water and the density of wildlife populations.

The stratification of sampling was performed independently of the bioregion classification and was based on the numbers of registered dogs per canton. However, the stratification naturally reflected the significant regional differences in density of the canine dog population, with the highest population density in the Plateau area (around 75% of the human population and more than 50% of the canine population) and the lowest in the central alpine regions. As a consequence, the estimates of seroprevalence in the Alpine regions of the country were based on much smaller

numbers than in the Plateau and Jura regions, potentially leading to a higher margin of error for the lower populated areas.

The prevalence of anti-leptospiral serum antibodies varied significantly between different bioregions. While the seroprevalence in the western high alpine regions (6.7%) and the south side of the Alps (10%) was low, the seroprevalence in the Northern half of the country which includes the Plateau, Jura and Northern Alps, ranged between 25.2% and 28.8% and odds ratios for seropositivity in these areas were high. The higher seroprevalence in bioregions with lower altitudes is biologically plausible due to less extreme climatic conditions throughout the year. Periods of extreme cold or extreme hot can be associated with reduced availability of surface water and reduced seropositivity rates, because leptospires are killed by freezing or desiccation (Adler, 2015). In addition to a milder climate, lower Switzerland has a large number of lakes and rivers, which could facilitate indirect transmission of *Leptospira*. However, none of the variables such as “swimming” and “drinking from puddles” assessing exposure to surface water as potential risk factors for seropositivity remained significant in the multivariate analysis.

The higher seroprevalence in this cohort of dogs not suspected to have leptospirosis in the Jura and Plateau area correlates with previous studies describing the geographic distribution of dogs with acute clinical infection in Switzerland with 75.5% of dogs originating from the Plateau area (Major et al., 2014). A cluster of acute disease in humans was recently described and could be traced back to a surfing spot in canton Aargau, which is also situated in the plateau area (Schreiber et al., 2015). Based on these studies, the plateau region which includes numerous lakes and rivers appears to carry this highest risk of infection in Switzerland.

Dogs had 4.7 times higher odds of being seropositive to serovar Bratislava (MAT ≥1:100) if the owner also owned a horse. While horses can get infected with a wide range of leptospiral serovars, globally, reactivity to serovar Bratislava is most common and the horse is considered a maintenance host for this serovar (Ellis et al., 1983; Baverud et al., 2009; Adler, 2015; Arent et al., 2015). In a recent serologic survey from Switzerland, 58.5% of 615 tested horses showed MAT seropositivity to at least one serogroup, indicating current or recent infection. The most frequent reactive serovars were Pyrogenes (22.6%), Canicola (22.1%), Australis (19.2%) and Bratislava (15.9%) (Blatti et al., 2011). Horses could therefore be the source of exposure for dogs to serovar Bratislava. However, serovar Bratislava has also been isolated from other animals including badgers (Hathaway et al., 1983), hedgehogs, rats and sheep (Arent et al., 2016). Therefore other factors such as spending more time in nature and potentially increased contact with reservoir hosts while accompanying the owner on excursions on horseback could be equally important factors for dogs to get exposed to this serovar.

Dogs had 8 times higher odds of having a positive titre to serovar Grippotyphosa (MAT ≥1:100) if they were hunting. This association may be explained by increased contact to environmental sources and reservoir hosts of *Leptospira* and correlates with the higher odds for acute leptospirosis in dogs exposed to wild animals (Ghneim *et al.*, 2007). Hunting as a risk factor for leptospirosis is also supported by human data showing a high seroprevalence of leptospirosis in human hunters (Deutz et al., 2003a; Deutz et al., 2003b).

In this cohort, the odds of MAT seropositivity increased with increasing age. A correlation between age and MAT seropositivity has been shown in many other studies in horses (Kitson-Piggot and Prescott, 1987; Baverud et al., 2009; Blatti et al., 2011)

and dogs (Harland et al., 2013). This relationship is plausible as infection related antibodies can persist for years and the probability of having come in contact with an infectious agent increases over time.

More difficult to explain is the fact that being castrated was a significant risk factor for the seropositivity to serovar Australis (MAT ≥1:100). This finding is in contrast to previous studies showing that uncastrated dogs are overrepresented in cohorts of animals with acute clinical leptospirosis which lead to the hypothesis that sexually intact animals manifest more often risky behaviours like sniffing around and marking their territories (Ward et al., 2002; Major et al., 2014). However, due to the confounding influence of vaccination on this variable, the association between neuter status and seropositivity to serovar Australis should not be over interpreted.

A significant association between the living environment and seroprevalence was not found in this cohort of dogs. While and increased seroprevalence was found in some studies for dogs living in rural compared to urban areas (Ghneim et al. 2006), a recent meta-analysis identified a higher risk in urban environments (Azocar-Aedo & Monti, 2016). This is not surprising, as wildlife reservoirs can be found with high density in both urban and rural environments and represent a likely source of infection in both settings. Risk factors for exposure to pathogenic *Leptospira* identified in other studies include being male or living in a kennel (Scanziani et al., 2002), a higher risk of infection in mixed and working breeds (Harland et al., 2013; Azocar-Aedo and Monti, 2016) as well as in dogs used to swimming and drinking from outdoor sources of water (Ghneim *et al.,* 2007). None of these risk factors was confirmed in this cohort of dogs.

4.4. MAT, co-reactivities, potential confounding effects of vaccination

In the past the MAT has been widely used to determine previous exposure to *Leptospira* in domestic and wild animals and in humans (Adler, 2015). With this test,

the presence of anti-leptospiral IgG and/or IgM is determined based on agglutination of live *Leptospira* species after incubation with patient serum at various dilutions. The MAT relies not only on inclusion of a representative panel of *Leptospira* spp., but its interpretation also has an element of subjectivity, which can explain the significant variability noted when MAT results from the same patient are compared across different laboratories (Miller et al., 2011).

MAT has limited specificity with regards to the identification of the infecting serogroup in acute infection as patient IgM, which predominates in early infection, has lower binding specificity than IgG (Chernukha et al., 1976). Although not supported by evidence, it is assumed that IgG predominates in sera of patients that have previously been exposed thus providing more specific information on MAT serogroup specific reactivity patterns than acute sera. However, cross-reactivity between different serogroups can occur and lead to MAT reactivity at lower dilutions.

While the MAT is far from being a perfect method, it is currently the only tool available to document exposure in dogs not considered to be acutely infected (and not actively shedding). Despite its limitations the MAT is well accepted and extensively used to derive information about infecting serogroups in acutely sick (Boutilier et al., 2003; Ward et al., 2004; Ghneim et al., 2007; Hennebelle et al., 2013; Mayer-Scholl et al., 2013; Major et al., 2014) and in asymptomatic dogs (Davis et al., 2008; Arent et al., 2013; Schuller et al., 2015; Llewellyn et al., 2016). MAT-derived serogroup specific information has also been used to inform recommendations regarding the inclusion of additional serogroups into current anti-leptospiral vaccines (Ellis, 2010).

The authors are not aware of any study in dogs, correlating infecting serovar and MAT serogroup reactivity. Data in this respect is missing as it would so far have required culture and isolation of *Leptospira* from infected dogs, which is notoriously difficult. As

molecular typing tools that do not require previous leptospiral culture are being developed, those methods will allow estimating the precision of serogroup determination via MAT in dogs, and will be a very powerful tool to type *Leptospira* causing acute infection in dogs. For this project we had planned to complement the serologic data with molecular typing of *Leptospira* spp derived from PCR positive urine. This part of the study would have added very valuable additional information to be correlated with serologic data. However, due to the very low rate of shedding, this part of the project could not be pursued further.

The sensitivity and specificity of MAT in convalescent sera of dogs after acute clinical infection has been reported to be 100% and 92% respectively at a cut off titre of ≥1:800. However, this is of limited relevance to this population of dogs without clinical signs of acute leptospirosis. The sensitivity and specificity of the MAT in detecting chronic subclinical infection or post exposure titres has not been reported. The estimated prevalence of 10% used for power calculation was chosen after review of the recent literature reporting seroprevalence rates between 6% in dogs in Ireland using a MAT cut-off of ≥1:10 (Schuller et al., 2015), 11.4% in Greece (Burriel et al., 2003), 17% in dogs in southern Germany (Llewellyn et al., 2016), 17.1% in Washington State (Davis et al., 2008), 21.5% in Poland (Krawczyk, 2005) and 49% in kenneled dogs in Italy using all a cut off ≥1:100 (Scanziani et al., 2002). In the absence of a consensus of what represents an ideal cut off titres for MAT positivity, the decision was made to use two different cut offs (≥1:40 and ≥1:100) to calculated seroprevalence in this present study.

As expected, co-reactivities were more common at lower titres. Most common co-reactivities were those between serogroups contained in the current vaccines and could therefore be related to vaccination. The MAT does not discriminate between

vaccination titres and titres due to exposure, thus adding further difficulty to the interpretation of canine tests, as a large proportion of dogs are vaccinated with either bivalent or quadrivalent whole cell anti-leptospiral vaccines. Despite all these limitations we decided to use MAT to estimate the seroprevalence in dogs not suspected to have leptospirosis in Switzerland and to apply statistical methods to minimise the effect of vaccination on prevalence results. In the absence of specific data regarding antibody titres after vaccination with a quadrivalent vaccine available on the European market at the time of the planning of the study, the results of a study describing the dynamics of antibody titres after vaccination with anti-leptospiral vaccines available on the US market were used to define inclusion criteria with regards to vaccination. In this study the majority of dogs developed positive MAT titers immediately after vaccination, a minority of them remained seropositive by week 15 (Martin et al., 2014). On the basis of these results, we decided to select dogs vaccinated at least 16 weeks (112 days) before testing. Due to the multicentre design of this study, this inclusion criterion was however violated in 10% of dogs. Subgroup analysis between vaccinated and unvaccinated dogs, as well as dogs not vaccinated in the past 112 days showed that albeit seroprevalence was slightly lower if recently vaccinated dogs were excluded, there was good correlation with regards to the reactive serogroups, in particular if considering the MAT titres ≥1:100.Therefore the decision was made to perform risk factor analysis on the entire cohort, regardless of vaccination status and to introduce the variable “days since vaccination” as a risk factor in the model to account for the possible confounding effect of vaccination. This variable only remained significant as an effect modifier for the associations between age and MAT seropositivity and for the association between castration and MAT seropositivity. We are therefore confident that the presented risk factors reflect risk factors associated with natural infection and not vaccination.

In order to describe titre dynamics in dogs with MAT titres ≥1:40 follow up testing 1-2 weeks later was advised. However, a large proportion of dogs was tested immediately prior to vaccination precluding that information of relevance to this study could be retrieved from retesting. Therefore only a small proportion of dogs was retested and results consistent with recent infection was found in 18% (2/11) of these dogs.

4.5. Urinary shedding

One of the hypotheses tested in this study was that dogs contribute to the maintenance and spread of pathogenic *Leptospira* in the environment by shedding *Leptospira* in their urine. In that regard urine samples from 408 dogs were tested by qPCR. Care was taken to preserve leptospiral DNA during shipment by use of a stabiliser, which had shown to preserve leptospiral DNA for at least 48 h post sampling. However, only 1/408 samples tested positive. In this dog contact to small rodents was the likely source of infection however and material was insufficient for molecular typing of the serovar or species level. Based on these results we concluded that urinary shedding by asymptomatic dogs is uncommon. This could be explained by the fact that dogs, albeit being an important incidental host for serogroup Australis in Switzerland, does not appear to be an efficient maintenance host for any of the locally prevalent strains, not included in the current vaccines. However, the influence of intermittent shedding and lack of detection due to pre-analytical or analytical factors also need to be considered and could potentially lead to a large underestimation of the actual level of renal carriage of pathogenic *Leptospira* in the canine population (Gay et al., 2014).

5. Conclusions

In conclusion, the results of this study show that dogs in Switzerland are commonly exposed to pathogenic *Leptospira* spp. with exposure being most common to serovars belonging to serogroup Australis. The Swiss Jura and plateau regions carry the highest

risk of infection. Contact to horses and hunting were significant risk factors associated with seroprositivity to serogroups Bratislava and Gripotyphosa respectively. Based on our findings vaccination of dogs with quadrivalent vaccines containing serogroups Australis and Gripotyphosa in addition to serogroups Icterohaemorrhagiae and Canicola is advised. The risk of dogs contributing to the spread of pathogenic *Leptospira* in the environment appears low.

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Figure legends

Figure 1. Graphical overview of stdy population and statistical analyses MAT: Microscopic agglutination test; RT-PCR: *LipL32* real time PCR

AUS: Australis; CAN: Canicola, GRI: Grippotyphosa, ICT: Icterohaemorrhagie

Figure 2. The biogeographic regions of Switzerland defined by Gonseth and Wohlgemuth (2001). The six biogeographic regions of Switzerland and the number of MAT seropositive dogs per total tested at a cut-off ≥ 1:40 and ≥ 1:100 are shown.

Figure 3. **The geographical distribution of 377 dogs tested for serum antibodies against *Leptospira* spp via MAT.** Geocoding was performed based on the zip code of the owner's home address. Seropositivity was defined as positive MAT titre at ≥1:100. Borders indicate the biogeographic regions of Switzerland defined by Gonseth and Wohlgemuth (2001).

Figure 4: Relative prevalence (%) of MAT reactivity to any serovar (All) and to individual serovars at reciprocal titres of ≥1:100 (A, C) and ≥1:40 (B, D). Prevalence is shown for vaccinated and non-vaccinated dogs (A-B), as well as between dogs vaccinated with a bi-valent and quadrivalent anti-leptospiral vaccines (C, D). Tested serovars: Australis (AUS), Autumnalis (AUT), Ballum (BAL), Bratislava (BRA), Canicola (CAN), Copenhageni (COP), Grippotyphosa (GRI), Hardjo (HAR), Icterohaemorrhagie (ICT), Pomona (POM), Pyrogenes (PYR) and Tarassovi (TAR).

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Tables

Table 1. Panel of 12 *Leptospira* spp. used as live antigens for Microscopic Agglutination testing (MAT).

Genomospecies	Serogroup	Serovar	Strain
L.interrogans	Australis	Australis	Ballico
	Australis	Bratislava	Jez-Bratislava
	Autumnalis	Autumnalis	Akiyami
	Canicola	Canicola	Hond Utrecht IV
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
	Icterohaemorrhagiae	Copenhageni	M20
	Pomona	Pomona	Pomona
	Pyrogenes	Pyrogenes	Salinem
	Sejroe	Hardjo	Hardjoprajitno
L.borgpetersenii	Ballum	Ballum	Mus127
	Tarassovi	Tarassovi	Perepelitsin
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V

Table 2. Frequency of variables collected via questionnaire for dogs tested for anti-leptospiral serum antibodies via MAT.

Variable	Category	n	%	95%CI
Vaccine	Not vaccinated	84	22.5%	18.2-26.5
	Bivalent	43	11.5%	8.3-14.7
	Quadrivalent	246	66%	61.1-71
Sex	Female	170	46.1%	41.2-51.2
	Male	199	53.9%	48.8-58.8
Castrated	No	168	45.7%	40.6-50.7
	Yes	200	54.3%	49.3-59.4
Other animals in the household	No	107	31.2%	26.3-36.1
	Dog	117	34.1%	29.1-39.1
	Cat	59	17.2%	13.2-21.2
	Dog and Cat	32	9.3%	6.3-12.4
	Livestock +/-Dog/cat	5	1.5%	0.2-2.7
	Horses +/- Dog/cat	23	6.7%	4.1-9.4
Bioregion	Jura	51	13.5%	10.1-16.7
	Central Plateau	230	61%	56.2-65.8
	Northern Alps	66	17.5%	13.8-21.5
	Central western Alps	15	4%	2.1-6.1
	Central Eastern Alps	5	1.3%	0.3-2.7
	Southern Alps	10	2.7%	1.3-4.5
Surrounding	Shelter	21	5.8%	3.6-8.3
	Urban	49	13.6%	10-17.2
	Rural	98	27.1%	23-31.9
	Farm	61	16.9%	13-20.8
	Urban + Garden	27	7.5%	5-10.2
	Rural + Garden	105	29.1%	24.4-33.8
Contact to acutely ill animals	No	162	47.9%	42.3-53.3
	Yes	28	8.3%	5.6-11.5
	I do not know	148	43.8%	38.2-49.1
Previously diagnosed and treated for Leptospirosis	No	330	97.6%	95.9-99.1
	Yes	8	2.4%	0.9-4.1

Fever this past year (dog)	No	305	90.2%	87-93.5
	Yes	33	9.8%	6.5-13
Fever this past year (owner)	No	270	81.1%	77.2-85.3
	Yes	63	18.9%	14.7-22.8
Outside	<50% of time	259	75.7%	71.3-80.1
	>50% of time	83	24.3%	17.6-31.3
Spends time in forests	No	31	9.2%	6.2-12.4
	Yes	307	90.8%	87.6-93.8
Drinking from puddles	No	104	30.9%	26.1-35.9
	Yes	233	60.1%	64.1-73.9
Swimming	No	110	32.7%	27.7-37.8
	Yes	226	67.3%	62.2-72.3
Contacts to wild animals	No	190	56.2%	50.6-61.5
	Yes	94	27.8%	23.4-32.5
	I do not know	54	16%	12.1-20.1
Hunting	No	326	96.4%	94.4-98.2
	Yes	12	3.6%	1.8-5.6

Table 3. Number of individuals, relative prevalence and 95% CI per *Leptospira* serovar for all dogs (n=377). Tested serovars: Australis (AUS), Autumnalis (AUT), Ballum (BAL), Bratislava (BRA), Canicola (CAN), Copenhageni (COP), Grippotyphosa (GRI), Hardjo (HAR), Icterohaemorrhagie (ICT), Pomona (POM), Pyrogenes (PYR) and Tarassovi (TAR).

MAT Titer		All Serovars	AUS	BRA	COP	CAN	GRI	ICT	POM	PYR	TAR	AUT	BAL	HAR
≥1:20	n	276	236	76	115	170	60	64	68	22	9	64	51	2
	%	73.2	62.6	20.2	30.5	45.1	15.9	17	18	5.8	2.4	17	13.5	0.5
	CI	69-77.7	57.8-67.6	15.9-24.4	26-35.3	40.1-50.1	11.9-19.9	13.3-20.7	14.3-22	3.4-8.5	1.1-4	13.3-21	10.1-17	0-1.3
≥1:40	n	210	123	54	69	94	43	36	37	7	2	27	3	1
	%	55.7	32.6	14.3	18.3	24.9	11.4	9.5	9.8	1.9	0.5	7.2	0.8	0.3
	CI	50.4-61	28.1-37.4	10.6-18	14.3-22.5	20.7-29.4	8.2-14.9	6.9-12.7	6.9-13	0.5-3.2	0-1.3	4.5-9.8	0-1.9	0-0.8
≥1:100	n	94	56	33	23	19	17	6	15	3	1	10	-	-
	%	24.9	14.9	8.8	6.1	5	4.5	1.6	4	0.8	0.3	2.7	-	-
	CI	20.4-29.4	11.4-18.6	6.1-11.7	3.7-8.5	2.9-7.4	2.7-6.9	0.5-2.9	2.1-6.1	0-1.9	0-0.8	1.3-4.2	-	-
≥1:200	n	46	21	20	11	7	9	3	6	2	-	5	-	-
	%	12.2	5.6	5.3	2.9	1.9	2.4	0.8	1.6	0.5	-	1.3	-	-
	CI	9-15.6	3.4-8	3.2-7.7	1.3-4.8	0.5-3.4	1.1-4	0-1.9	0.5-2.9	0-1.3	-	0.3-2.7	-	-
≥1:400	n	23	9	11	3	-	1	1	2	-	-	3	-	-
	%	6.1	2.4	2.9	0.8	-	0.	0.3	0.5	-	-	0.8	-	-
	CI	4-8.8	1.1-4	1.3-4.5	0-1.9	-	0-0.8	0-0.8	0-1.3	-	-	0-1.9	-	-
≥1:800	n	8	5	5	-	-	1	-	1	-	-	1	-	-
	%	2.1	1.3	1.3	-	-	0.3	-	0.3	-	-	0.3	-	-
	CI	0.8-3.7	0.3-2.7	0.3-2.7	-	-	0-0.8	-	0-0.8	-	-	0-0.8	-	-
≥1:1600	n	6	4	1	-	-	-	-	-	-	-	1	-	-
	%	1.6	1.1	0.3	-	-	-	-	-	-	-	0.3	-	-
	CI	0.5-2.9	0.3-2.1	0-0.8	-	-	-	-	-	-	-	0-0.8	-	-
≥1:3200	n	2	1	-	-	-	-	-	-	-	-	-	-	-
	%	0.5	0.3	-	-	-	-	-	-	-	-	-	-	-
	CI	0-1.3	0-0.8	-	-	-	-	-	-	-	-	-	-	-

Table 4. Number of individuals, relative prevalence and 95% CI per *Leptospira* serovar for unvaccinated dogs (n=84). Tested serovars: Australis (AUS), Autumnalis (AUT), Ballum (BAL), Bratislava (BRA), Canicola (CAN), Copenhageni (COP), Grippotyphosa (GRI), Hardjo (HAR), Icterohaemorrhagie (ICT), Pomona (POM), Pyrogenes (PYR) and Tarassovi (TAR).

MAT Titer		All Serovars	AUS	BRA	COP	CAN	GRI	ICT	POM	PYR	TAR	AUT	BAL	HAR
≥1:20	n	55	43	13	19	22	6	6	10	5	4	14	10	1
	%	65.5	51.2	15.5	22.6	26.2	7.1	7.1	11.9	6	4.8	16.7	11.9	1.2
	CI	54.8-75	40.5-61.9	8.3-23.8	14.3-31	16.7-35.7	2.4-13.1	2.4-13.1	6-19	1.2-11.9	1.2-9.5	9.5-25	6-19	0-3.6
≥1:40	n	37	17	11	9	11	5	3	4	-	1	7	1	1
	%	44	20.2	13.1	10.7	13.1	6	3.6	4.8	-	1.2	8.3	1.2	1.2
	CI	33.3-53.6	11.9-28.6	6-20.2	4.8-17.9	6-20.2	1.2-11.9	0-8.3	1.2-9.5	-	0-3.6	3.6-14.3	0-3.6	0-3.6
≥1:100	n	15	8	7	3	3	3	-	2	-	-	2	-	-
	%	17.9	9.5	8.3	3.6	3.6	3.6	-	2.4	-	-	2.4	-	-
	CI	10.7-26.2	3.6-16.6	2.4-14.3	0-8.3	0-8.3	0-8.3	-	0-6	-	-	0-6	-	-
≥1:200	n	10	4	5	2	1	3	-	1	-	-	2	-	-
	%	11.9	4.8	6	2.4	1.2	3.6	-	1.2	-	-	2.4	-	-
	CI	6-19	1.2-9.5	1.2-10.7	0-6	0-3.6	0-8.3	-	0-3.6	-	-	0-6	-	-
≥1:400	n	4	-	2	-	-	-	-	1	-	-	1	-	-
	%	4.8	-	2.4	-	-	-	-	1.2	-	-	1.2	-	-
	CI	1.2-9.5	-	0-6	-	-	-	-	0-3.6	-	-	0-3.6	-	-
≥1:800	n	1	-	1	-	-	-	-	-	-	-	1	-	-
	%	1.2	-	1.2	-	-	-	-	-	-	-	1.2	-	-
	CI	0-3.6	-	0-3.6	-	-	-	-	-	-	-	0-3.6	-	-
≥1:1600	n	1	-	-	-	-	-	-	-	-	-	1	-	-
	%	1.2	-	-	-	-	-	-	-	-	-	1.2	-	-
	CI	0-3.6	-	-	-	-	-	-	-	-	-	0-3.6	-	-
≥1:3200	n	2		-	-	-	-	-	-	-	-	-	-	-
	%	0.7		-	-	-	-	-	-	-	-	-	-	-
	CI	0-1.7		-	-	-	-	-	-	-	-	-	-	-

Table 5. Number of individuals positive against one, two or more serogroups at a MAT Titre of ≥1:40 and MAT ≥1:100.

N° of positive Serogroups	MAT ≥1:40 n (%)	MAT ≥1:100 n (%)
1	21 (23.1)	58 (62.4)
2	30 (33.0)	19 (20.4)
3	25 (27.5)	8 (8.6)
4	7 (7.7)	6 (6.5)
5	7 (7.7)	1 (6.5)
6	1 (1.1)	1 (1.1)

Table 6. Risk factors associated with a MAT positivity.

Variable	ALL40	ALL100	AUS100	BRA100	GRI100	CAN100	COP100
Swimming	*						
Castrated		*	**				
Lives with other animals	*			**		*	*
Hunting					**		
Outside >50% of time							*
Bioregion	**	*					
Surroundings	*						
Forest	*						
Season	*						
Age	**	**					
Days since last vaccination	**	**	**	*			*

* Univariate analysis ($p \leq 0.2$)

** Multivariate analysis ($p < 0.05$)

Outcomes: Seropositivity in dogs against any of the tested serovars at MAT cut-offs ≥1:40 (ALL40) and ≥1:100 (ALL100), seropositivity in dogs at the MAT cut-off ≥1:100 for serovars Australis (AUS100), Bratislava (BRA100), Grippotyphosa (GRI100), Canicola (CAN100) and Copenhageni (COP100).

Table 7. Statistically significant risk factors for seropositivity against any serovar at a MAT cut-off ≥ 1:40 tested in a multivariable logistic regression model.

Covariate	Levels	Odds ratio	95%CI	p-value
Bioregion	6[#]	Ref.		
	1	11.17	1.69-226.72	0.034*
	2	12.43	2.04-244.12	0.023*
	3	14.51	2.23-292.71	0.018*
	4	5.53	0.63-125.11	0.17
	5	53.05	3.39-2378.75	0.012*
Age (years)		1.29	1.13-1.47	0.0001*
Days since vaccination		1.0008	0.9988-1.0028	0.42
Days since vaccination*Age		0.9995	0.9992-0.9998	0.0034*

* Statistically significant at $p < 0.05$, AIC full model = 408.25 & n=301, AIC final model = 458.49 & n=347.

[#] The numbers correspond with the bioregions (1) Jura, (2) Central Plateau, (3) Northern Alps, (4) Central Western Alps, (5) Central Eastern Alps and (6) Southern Alps.

Table 8. Statistically significant risk factors for seropositivity against any serovar and against serovars Australis, Bratislava and Grippotyphosa at a MAT cut-off ≥ 1:100 tested in multivariable logistic regression models. Associations were considered significant if $p<0.05$. The Akaike information criterion (AIC) is used to indicate the relative quality of the used model.

Covariate	Levels	Odds ratio	95%CI	p-value
Risk factors for seropositivity against any serovar at MAT cut-off ≥ 1:100[&]				
Days since vaccination		1.0011	0.9986-1.0034	0.39
Age (years)		1.25	1.09-1.43	0.0012*
Days since vaccination*Age		0.9995	0.9991-0.9999	0.0077*
Risk factors for seropositivity against serovar Australis at a MAT cut off ≥ 1:100[Δ]				
Castrated	No	Ref		
	Yes	1.98	1.08-3.74	0.030*
Days since vaccination		0.9969	0.995- 0.998	0.0004*
Risk factors for seropositivity against serovar Bratislava at a MAT cut-off ≥ 1:100[▾]				
Dog		1.40	0.49-4.32	0.53
Cat		2.27	0.72-7.37	0.16
Dog and Cat		2.40	0.58-9.02	0.20
Livestock +/-Dog/cat		4.21	0.20-34.77	0.23
Horses +/- Dog/cat		4.68	1.23-17.19	0.019*
Risk factors for seropositivity against serovar Grippotyphosa at a MAT cut-off ≥ 1:100[#]				
Hunting		8.03	1.64-30.82	0.004*

[&] AIC full model = 361.64 & n=319, AIC final model = 382.36 & n=347.

[Δ] AIC full & final model = 296.83 & n=367

[▾]AIC full model = 218.26 & n=342, AIC final model = 217.87, n=342

[#]AIC full & final model = 126.74 & n=337.